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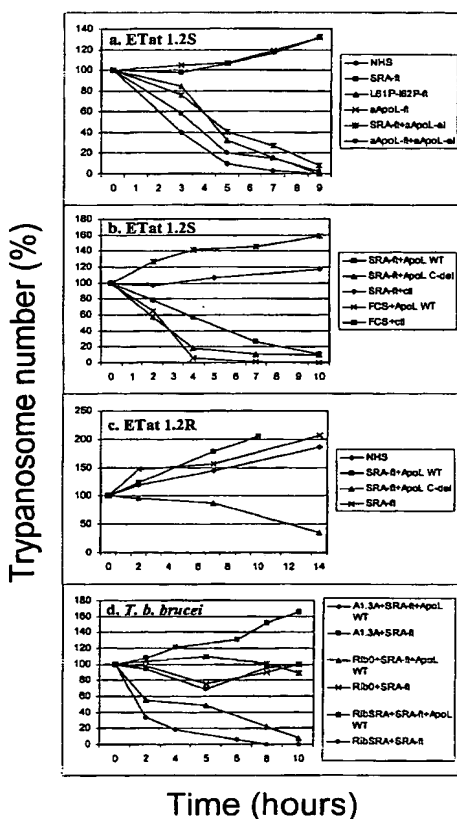
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(54) Title: **APOLIPOPROTEIN L-I AND/OR DERIVATED POLYPEPTIDE FOR THE TREATMENT OF AND/OR THE PREVENTION OF DISEASES INDUCED BY TRYPANOSOMA**



(57) Abstract: The present invention is related to a composition comprising apolipoprotein L-I, the use of apolipoprotein L-I or a derivated polypeptide for the diagnostic, the treatment and/or the prevention of diseases induced in mammals by Trypanosoma. Another aspect is related to a transgenic non-human mammal comprising a polynucleotide expressing said apolipoprotein or a derivated polypeptide and which is tolerant or resistant to said Trypanosoma infection.



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APOLIPOPROTEIN L-I AND/OR A DERIVATED POLYPEPTIDE FOR THE
TREATMENT AND/OR THE PREVENTION OF DISEASES INDUCED BY
TRYPANOSOMA

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Field of the invention

The present invention is related to the use of apolipoprotein L-I for the treatment and/or the prevention of diseases induced in mammals by Trypanosoma, especially African Trypanosoma.

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The present invention is also related to a derivated polypeptide of said apolipoprotein L-I sequence which could also be used for therapeutic and/or diagnostic applications directed against Trypanosoma, especially African Trypanosoma.

20

A last aspect of the present invention is related to a transgenic bovidae which is able to produce naturally said apolipoprotein L-I or a polypeptide derivated from said apolipoprotein L-I sequence and which is tolerant or resistant to said Trypanosoma infection.

25

Background of the invention

Trypanosoma brucei brucei infects a wide range of mammals but is unable to infect humans because this *T. brucei* subspecies is lysed by NHS^{1,2}. In contrast, *Trypanosoma brucei rhodesiense* exhibits sensitivity or resistance to normal human serum (NHS), depending on antigenic variation. The latter phenotype arises because the gene encoding resistance, serum resistance associated

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protein (SRA), is present in a single of the multiple polycistronic units where the genes for the variant antigen, the variant surface glycoprotein (VSG), can be transcribed ⁴. The product of serum resistance associated
5 protein (SRA) is an atypical variant surface glycoprotein (VSG), shorter than average (410 aa instead of approximately 490) ³. Anti-SRA antibodies detected a protein around 50 kDa (Fig. 1 lanes 1,2), which accumulates in the lysosome as revealed with specific markers for the
10 endocytic compartment, namely tomato lectin (flagellar pocket, endosomes and lysosome) ⁵ and antibodies against the lysosomal membrane glycoprotein p67 ⁶ (Fig. 2a,b).

Summary of the invention

15 A first aspect of the present invention is related to the use of a molecule or a pharmaceutical composition (including a vaccine) comprising said molecule and an adequate pharmaceutical carrier or diluent for the manufacture of a medicament in the treatment and/or the
20 prevention of mammals diseases induced by *Trypanosoma*, especially African *Trypanosoma* selected from a group consisting of *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense* and, *Trypanosoma gambiense*.

The specific molecule used according to the
25 invention is the apolipoprotein L-I polypeptide, preferably the human apolipoprotein L-I (ApoL-I) or its derivatives: an homologue polypeptide (polypeptide which exists in other species than the human species), pharmaceutical active fragments thereof, variants thereof, a polynucleotide
30 encoding said polypeptide(s), its homologues, fragments or variants or a cell transformed by said polynucleotide and expressing said polypeptide or fragments. The human apolipoprotein L-I polypeptide and polynucleotide correspond to the sequence SEQ.ID.N°1. The preferred

pharmaceutical active fragments are the sequences starting from amino acid 1 up to amino acid 342, the sequence starting from the amino acid 343 up to the amino acid 398, the sequence starting from the amino acid 340 up to the amino acid 362 and the sequence starting from the amino acid 356 up to the amino acid 398.

The molecule used according to the invention can also be an inhibitor directed against said apolipoprotein L-I, such as the Trypanosoma polypeptide SRA, a pharmaceutical active fragment thereof (binding specifically the ApoL-I polypeptide) or any molecule which may mimic the interaction between the polypeptide SRA and the apolipoprotein L-I. For instance, such inhibitor which may mimic said interaction is an antibody directed against the specific apolipoprotein L-I (or a hyper variable portion thereof), especially directed against the ApoL-I terminal fragment which interact with the polypeptide SRA. Such inhibitor could be a monoclonal or a polyclonal antibody.

A last aspect of the present invention is a monoclonal or polyclonal anti-idiotypic antibody (or a hyper variable portion thereof) directed against the anti-apolipoprotein L-I antibody above described which may mimic the structure of a fragment of said apolipoprotein L-I (epitope which interacts with the polypeptide SRA). The molecule of the invention can also be a complex between said elements (complex between fragments, complex of antibody-fragment, etc).

These various polypeptides, polynucleotides, variants, fragments or inhibitors could be used for a therapeutic (administration of ApoL-I or its derivatives), prophylactic (vaccine comprising the inhibitor (SRA or a fragment or SRA polypeptide)) or diagnostic (detection of ApoL-I or SRA by inhibitors directed against them) purpose

in order to identify, treat and/or prevent infections caused by Trypanosoma in a mammal (especially in a bovidae or a human).

The scope of protection extends also to said
5 various polypeptides, polynucleotides, variants, fragments or inhibitors as such for any application.

The various molecules according to the invention could be also used in diagnostic kits for characterizing the immune reaction of a mammal regarding
10 the administration of the molecule or the pharmaceutical composition (vaccine) according to the invention and for the detection or the monitoring of inhibitors production by a mammal.

Furthermore, the various molecules according
15 to the invention could also be used for the purification of apolipoprotein L-I, for instance by using one or more of said inhibitors (SRA polypeptide, fragment of SRA polypeptide or antibody directed against the ApoL-I) in a chromatographic column or another purification apparatus.

20 A last aspect of the present invention is related to a non-human genetically modified mammal, in particular a genetically modified bovid, more particularly a genetically modified cow (obtained by the method well-known by the person skilled in the art), which is
25 expressing a polynucleotide encoding apolipoprotein L-I, preferably the human apolipoprotein L-I, a homologue of said polypeptide, a fragment thereof or a variant thereof, more preferably the fragment having the sequence starting from the amino acid 1 up to amino acid 342.

30 Advantageously, said polynucleotide sequence expressing the polypeptide apolipoprotein L-I, its homologue, its fragment or its variant, is under the control of transcription promoter, specifically expressed in endothelial cells.

Advantageously, said non-human genetically modified mammal is resistant to Trypanosoma infections, especially diseases induced by African trypanosomes (Nagana).

5 The present invention will be described in more details in the following detailed description of the invention in reference to the enclosed figures.

Definitions

10 "Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or
15 oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical
20 modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid
25 side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched
30 as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-

ribosylation, amidation, covalent attachment of flavin, covalent attachment of a hem moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-linkings, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Comany, New York, 1993 and Wolt, F., Posttranslational Protein Modifications: Perspectives and Prospects, pp. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al., "Analysis for protein modifications and nonprotein cofactors", *Meth. Enzymol.* (1990) 182 : 626-646 and Rattan et al., "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci* (1992) 663 : 48-62.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "Polynucleotide" refers to triple-stranded

regions comprising RNA or DNA or both RNA and DNA. The term "Polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "Polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions (preferably conservative), additions and deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an

allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis. Variants should retain
5 one or more of the biological activities of the reference polypeptide. For instance, they should have similar antigenic or immunogenic activities as the reference polypeptide. Antigenicity can be tested using standard immunoblot experiments, preferably using polyclonal sera
10 against the reference polypeptide. The immunogenicity can be tested by measuring antibody responses (using polyclonal sera generated against the variant polypeptide) against purified reference polypeptide in a standard ELISA test. Preferably, a variant would retain all of the above
15 biological activities.

The variant will preferably present an identity of sequence higher than 70%, 75%, 80%, 85%, 90%, 95%, 98%-99% with the original peptide (SEQ.ID.N°1).

"Identity" is a measure of the identity of
20 nucleotide sequences or amino acid sequences. In general, the sequences are aligned so that the highest order match is obtained. "Identify" per se has an art-recognised meaning and can be calculated using published techniques. See, e.g.: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M.,
25 ed., Oxford University Press, New York, 1988; BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, 1993; COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds, Humana Press, New Jersey, 1994; SEQUENCE ANALYSIS IN MOLECULAR
30 BIOLOGY, von Heijne, G., Academic Press, 1987; and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds, M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known

to skilled artisans (Carillo, H., and Lipton, D., *SIAM J Applied Math* (1998) 48 : 1073). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to those disclosed in Guide to
5 Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipton, D., *SIAM J Applied Math* (1988) 48 : 1073. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity
10 and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., *J Molec Biol* (1990) 215 : 403). Most preferably, the program used to determine identity levels was the GAP program, as was used in the Examples hereafter.

15 As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the
20 polynucleotide sequence may include an average up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of
25 the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may
30 occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

A fragment may be "free-standing" or comprised within a larger polypeptide of which they form a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-30, 31-60, 61-90, 91-120, 121-150, and 150, 200, 250 to the end of the polypeptide. In this context "about" includes the particularly recited ranges larger or smaller by several, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes.

Preferred fragments include, for example, truncated polypeptides having the amino acid sequence of the polypeptides, except for deletion of a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus and / or transmembrane region or deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred are fragments characterised by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Other preferred fragments are biologically active fragments.

30 Description of the drawings

The fig. 1 represents the pTSARib vector contained, or not (-), different versions of serum resistance associated protein (SRA) (WT = wild type; del = deleted of the indicated peptide; SRA/VSG = chimera

associating the SRA 1-192 and variant surface glycoprotein (VSG) 288-490 peptides). The cells were analysed by the human serum resistance test (HSRT) *in vivo* and *in vitro* ⁴, and qualified as resistant (R) or sensitive (S). The
5 transformants were grown in mice in the presence of foetal calf serum (FCS) or normal human serum (NHS). The significance of the 45 kDa band (asterisk) is unknown.

The fig. 2 represents the trypanosomes were analysed by confocal fluorescence microscopy after
10 incubation for 1 h at 37°C in the presence (c,d,e) or absence (a,b) of Alexa 594-apoL-I. DAPI, tomato lectin (TL) and anti-p67 monoclonal antibodies (p67) respectively label the DNA (large dot: nucleus, small dot: kinetoplast), endocytic compartment and lysosome ^{5,6}.

15 The fig. 3. represents a. Patterns of NHS proteins bound to resins containing either no protein (-), or different versions of His-SRA. NHS (lanes 1-8) or *in vitro* synthesized [³⁵S]-apoL-I (lanes 9,10) was incubated with the resin. Bound proteins were eluted with either
20 imidazole (lanes 1-4, 7-10) or deoxycholate (lanes 5,6), and revealed by Coomassie blue staining (lanes 1-6), binding of anti-apoL-I antibodies (lanes 7,8) or autoradiography (lanes 9,10). The double arrow designates bands specifically bound with functional SRA, and asterisks
25 label eluted His-SRA. b. Immunoprecipitation with anti-V5 antibodies, of *in vitro* synthesized [³⁵S]-SRA (48 kDa) incubated or not with [³⁵S]-V5-apoL-I (42 kDa). The predicted [³⁵S]-methionine ratio between these proteins is 4:9. c. Model of the serum resistance associated protein
30 (SRA) 31-79 fragment (dark blue ribbon) in anti-parallel interaction with the apoL-I 340-392 fragment (light blue ribbon). I58 (green), L61 (pink) and I62 (mauve) are in CPK representation. d. serum resistance associated protein (SRA) and apoL-I features mentioned in the text (SP=signal

peptide; HT=hydrophobic tail; broken lines symbolize the interactions between the α -helices).

The fig. 4 represents a. Incubation of ETat 1.2S with differently treated NHS (SRA-ft, L61P/I62P-ft, aApoL-ft = flow-through fraction from SRA-, L61P/I62P SRA- and anti-apoL-I-Sepharose, respectively; aApoL-el = eluate of the fraction bound to anti-apoL-I-Sepharose). b. Incubation of ETat 1.2S in either SRA-ft or FCS supplemented with recombinant apoL-I (WT = wild type; C-del = lacking the C-terminal 343-398 peptide) or with the equivalent fraction from control CHO cells (ctl). c. Incubation of ETat 1.2R in NHS or SRA-ft supplemented with recombinant apoL-I. d. Incubation of different *Trypanosoma brucei brucei* cell lines in serum resistance associated protein (SRA)-ft supplemented or not with recombinant apoL-I. The pTSARib transformants (Rib0, RibSRA) are pleomorphic forms that do not grow under these incubation conditions.

The fig. 5 represents the amino acid and nucleotide sequences of the human apolipoprotein polypeptide (SEQ.ID. N°1).

Detailed description of the invention

The inventors have discovered that Anti-SRA antibodies detect a protein around 50 kDa (Fig. 1 lanes 1,2), which accumulates in the *Trypanosoma* lysosome as revealed with specific markers for the endocytic compartment, namely tomato lectin (flagellar pocket, endosomes and lysosome) ⁵ and antibodies against the lysosomal membrane glycoprotein p67 ⁶ (Fig. 2a,b).

Since transfection of SRA allows *Trypanosoma brucei brucei* to grow in NHS ⁴ (Fig. 1 lanes 3-5) the inventors have investigated the minimal requirements of SRA necessary for resistance by generating trypanosomes

expressing various serum resistance associated protein (SRA) mutants (SRA scheme in Fig. 3d). These cells were analysed for serum resistance associated protein (SRA) expression and sensitivity to normal human serum (NHS) (Fig. 1).

Although serum resistance associated protein (SRA) contains N-linked high mannans, these were not involved in resistance since individual or collective replacement of the putative N-glycosylation sites did not affect SRA-mediated resistance (lanes 6,7). The C-terminus and GPI anchoring signal were also dispensable since truncated serum resistance associated protein (SRA) versions lacking this region still conferred resistance (lanes 8,9). Similarly, deletions of the N-terminal signal peptide or of the most hydrophobic region (aa 98-118) were without effect (lanes 10,11). Collectively these data mapped between aa 32-97 and/or 119-192 the minimal serum resistance associated protein (SRA) stretches required for resistance.

Since the former region contains distinctive features of the amphipathic α -helix involved in VSG dimerization, -e.g. heptad repeats ⁷, the inventors have introduced point mutations predicted to disrupt this helix. Two independent double mutations (L61P/I62P and I58Q/I62Q) abolished the resistance phenotype, whereas mutations in an adjacent region (V40Q/V43Q/L47Q) did not (lanes 12-14). Thus, the α -helix 54-65 containing the key hydrophobic residues I58, L61 and I62 was necessary to confer resistance.

1. Serum resistance associated protein (SRA) binds to Concanavalin A and contains N-linked high mannose glycans

5 Methods

Generation of transgenic trypanosomes expressing SRA mutants.

The serum resistance associated protein (SRA) gene in pTSARib-SRA⁴ was altered by site-directed
10 mutagenesis (Stratagene) and/or restriction endonuclease digestion and ligation with fragments from the AnTat 11.17 VSG¹⁷. The resultant plasmids were electroporated into *Trypanosoma brucei brucei* procyclic forms that were cyclically transmitted through tsetse flies to obtain
15 bloodstream form transformants⁴.

Production of serum resistance associated protein (SRA)

Serum resistance associated protein (SRA) was produced either by *in vitro* RNA translation using
20 BSDP1400HA65-SRA¹⁸, or by expression in *Escherichia coli* using pQE30 in which the 1-753 bp serum resistance associated protein (SRA) fragment was inserted between *Bam*HI and *Hind*III to encode a N-terminal His-tagged polypeptide. In the latter case, the protein was
25 solubilized from pellets of sonicated cell extracts, by incubation for 1 h at room temperature in 8 M urea, 2 mM mercaptoethanol, 0.1 M NaH₂PO₄, 10 mM Tris (pH 8), then dialysis against the same buffer containing successively 0.5 M urea, 0.35 M urea, 0.1 M urea and 1% CHAPS.

Antibodies

The anti-SRA antibodies were generated by rabbit immunization with the His-tagged 1-251 polypeptide, and affinity-purified by binding to antigen-coated nitrocellulose filters and acid elution. They were used after 1:2 and 1:100 dilution, for immunofluorescence and Western blot analysis, respectively. The anti-apoL-I antibodies⁸ were used at 1:20,000 dilution. Anti-p67 antibodies were from the Ali I-218 hybridoma supernatant (gift of D.G. Russell).

Interaction of serum resistance associated protein (SRA) with normal human serum (NHS)

Recombinant wild type or mutant His-SRA (100 µg) was incubated with 500 µl normal human serum (NHS) for 4 h at 4°C in 0.6 M NaCl, 0.35% CHAPS, 0.15 M Mes (pH 5.8) with a cocktail of protease inhibitors (complete EDTA-free including pepstatin, Roche) (buffer A). The mixture was then incubated for 30 min at 4°C with 100 µl of Ni-NTA beads (Qiagen). Processing of these beads, including elution of bound material with 250 mM imidazole, was performed in buffer A as described by Qiagen. Alternatively, His-SRA was covalently coupled to activated CH Sepharose 4B (Pharmacia), and the bound material was eluted in 5% Na deoxycholate, 50 mM Tris (pH 7.5).

Confocal fluorescence microscopy

The trypanosomes were fixed for 10 min in 3.7% paraformaldehyde and permeabilized for 10 min in 0.1% Triton X-100. Biotinylated tomato lectin, Alexa 488-streptavidin and Alexa 488/594-anti-mouse or anti-rabbit secondary antibodies were used. To detect apoL-I, the

deoxycholate eluate from SRA-Sepharose was coupled to Alexa 594 after extensive dialysis and depletion of serumalbumin by gel filtration, and incubated with trypanosomes for 1 h at 37°C. The samples were analyzed with a Leica TCS SP2
5 confocal microscope.

Production of native apoL-I

ApoL-I was obtained by elution of the normal human serum (NHS) material bound to anti-apoL-I-Sepharose, using 5% CHAPS, 0.1 M glycine (pH 2.8) followed by
10 neutralization with 1 M Tris (pH 8.0). Residual contaminants were identified by mass spectrometry.

Cloning of apoL-I

The apoL-I gene was amplified by RT-PCR using total RNA from HepG2 cells and the following pair of oligonucleotides:
Apol-F: 5'-TGTCCTCTGCGGTACCATGAGTGCACTTTTCCTTGGTGTGAGAG-3'; Apol-R: 5'-CCCTGCCCTGCTCGAGCAGTTCTTGGTCCGCCTGCAGAATC-3'. The 1,15
20 kb-fragment was digested by KpnI + XhoI and ligated with the KpnI/XhoI-digested pcDNA3.1/V5-HisA plasmid (Invitrogen). Various mutants were generated by site-directed mutagenesis (Stratagene), followed or not by deletions/ligations to remove specific fragments.

Serum resistance associated protein (SRA) contains three potential N-glycosylation sites and in order to determine whether the protein was glycosylated detergent extracts of *Trypanosoma brucei rhodesiense* were fractionated on Concanavalin A Sepharose. Serum resistance
25 associated protein (SRA) was clearly present in the material loaded onto the column but was not detectable in the flow through fractions, thus it was clear that serum resistance associated protein (SRA) is glycosylated (lanes
30 1,2). Interestingly, after elution with 0.3 M

methylemannoside only a doublet around 30 kDa was detected (lane 3). This 30 kDa doublet is also slightly visible in lane 1 and is sometimes, but not always, detected when probing Western blots of *Trypanosoma brucei rhodesiense* bloodstream form extracts with anti-SRA antibodies. It results very likely from endopeptidic cleavage near the centre of the protein. The 50 kDa doublet bound very tightly to the lectin and could not be eluted even with 0.6 M methylemannoside (data not shown). However, both the 50 kDa and the lower doublet were released when the resin was boiled in SDS PAGE sample buffer (lane 4). The 30 kDa material contained N-linked glycans since there was a slight decrease in size upon digestion with pNGaseF (compare lanes 5,6) or endoglycosidase H (compare lanes 5,7). The same decrease was observed when comparing the apparent electrophoretic mobility of wild type and N86D/N231D/N320D mutant of serum resistance associated protein (SRA) (Fig. 1, compare lanes 4-7). Taken together, these results clearly demonstrated that serum resistance associated protein (SRA) contains N-glycans of the high mannan type, as is the case for the C-terminal N-glycan of variant surface glycoprotein (VSG).

The finding that a α -helical region similar to that involved in the dimerization of VSGs was required for SRA-mediated resistance raised the possibility that serum resistance associated protein (SRA) neutralised trypanolytic constituents of normal human serum (NHS) via a coiled-coil interaction. Therefore, we subjected normal human serum (NHS) to affinity chromatography using an N-terminal His-tagged version of serum resistance associated protein (SRA) (aa 1-251) immobilized onto Ni-nitrilotriacetic acid (NTA) agarose. A 40 kDa doublet was specifically retained by serum resistance associated protein (SRA) (Fig. 3a lanes 1,2), and this occurred in

various conditions (pH 7.5 or 5.8, 0 or 0.6 M NaCl). Interestingly, the doublet was not observed using L61P/I62P serum resistance associated protein (SRA), the helix-disrupted version that does not confer resistance, whereas
5 it was still present with a functional SRA mutant (lanes 3,4). Similar results were obtained using serum resistance associated protein (SRA) covalently attached to Sepharose. In this case, the doublet was the only component bound, apart from a 65 kDa contaminant (lanes 5,6). Mass
10 spectrometry identified the 40 kDa doublet as apoL-I, a human apolipoprotein associated with HDL ⁸⁻¹¹ (the 65 kDa protein was serumalbumin). Anti-apoL-I antibodies confirmed this finding (lanes 7,8). The apoL-I gene was amplified by RT-PCR from RNA of HepG2 cells and cloned in an expression
15 vector. Various mutants were generated, concentrating on the sequence for a peptide (aa 343-355) exhibiting membrane-disrupting activity *in vitro*. In particular, the inventors have constructed two truncated versions ending respectively at aa 342 and 355 and a mutant with
20 compromised lipid-destabilising properties (L345Y/L352A/Y354L) ¹². [³⁵S]-wild type and mutant versions of apoL-I were synthesized *in vitro* and incubated with His-SRA. [³⁵S]-apoL-I bound to the serum resistance associated protein (SRA) as did the native protein (Fig. 3a lane 10).
25 No binding occurred using appropriate control resins (no protein or irrelevant His-tagged protein). Significantly [³⁵S]-apoL-I did not bind to one of the helix-disrupted mutants of serum resistance associated protein (SRA) (L61P/I62P, lane 9), and showed a significantly reduced
30 binding (50%) to the other (I58Q/I62Q). Deletion of the apoL-I C-terminus from aa 343 reduced the binding to 5% while the deletion mutant from aa 356 and the L345Y/L352A/Y354L point mutant showed 24% and 67% binding respectively.

These data indicated that the C-terminal region of apoL-I, which contains an amphipathic helix ⁸, is important for the interaction with serum resistance associated protein (SRA). The SRA/apoL-I interaction was confirmed by another approach, since incubation with V5-tagged apoL-I led to co-immunoprecipitation of serum resistance associated protein (SRA) by anti-V5 antibodies (Fig. 3b).

In addition, a synthetic peptide corresponding to the helix of serum resistance associated protein (SRA) (aa 54-65) appeared to interact directly with the 345-355 peptide of apoL-I since it abolished the liposome fusogenic activity of the latter. A model of the interacting helices was built on those of the variant surface glycoprotein (VSG) (Fig. 3c,d). The partners would be in anti-parallel configuration (-88 and -72 kcal/mol for the respective hydrophobic energy of the NC/CN and NC/NC matching, to be compared with -89 and -77 kcal/mol for the interaction between helices A and B in the reference variant surface glycoprotein (VSG) MiTat 1.2 ⁷ and in serum resistance associated protein (SRA), respectively). The phenotypic effect of the serum resistance associated protein (SRA) mutants could be explained by differences of complex stability, with -77 and -91 kcal/mol for the respective hydrophobic energy of the complexes between apoL-I and the I58Q/I62Q and V40Q/V43Q/L47Q SRA mutants, whereas in L61P/I62P the helical structure is broken and thus no stable complex can be formed.

The inventors have investigated the possible involvement of apoL-I in trypanosome lysis. ApoL-I was found to be internalised via the endocytic pathway (Fig. 2c), and to co-localize with serum resistance associated protein (SRA) in the lysosome (Fig. 2d,e). Fractionation of normal human serum (NHS) on serum resistance associated

protein (SRA)-Sepharose, which essentially retains apoL-I (Fig. 3a lanes 5,6), reproducibly resulted in total loss of lytic activity, as tested both *in vitro* (Fig. 4a) and in mice (see Methods). This result was not obtained with
5 resins devoid of serum resistance associated protein (SRA), or containing L61P/I62P SRA (Fig. 4a). Lytic activity was also lost when normal human serum (NHS) was fractionated on anti-apoL-I-Sepharose (Fig. 4a). As expected this resin retained apoL-I, together with various amounts of
10 serumalbumin, apolipoprotein J, amyloid protein P and transthyretin. Whereas elution of apoL-I from the serum resistance associated protein (SRA) column required high detergent concentrations, native apoL-I was easily eluted from anti-apoL-I Sepharose. Addition of this fraction to
15 sera depleted on either serum resistance associated protein (SRA)- or anti-apoL-I-Sepharose completely restored their lytic activity (Fig. 4a). To be certain that apoL-I was responsible for this reconstitution, we expressed recombinant His-apoL-I in CHO cells and added the purified
20 protein to normal human serum (NHS) depleted using SRA-Sepharose. The addition of physiological concentrations of recombinant apoL-I (around 8 $\mu\text{g/ml}$)⁸, fully restored the lytic activity on either *Trypanosoma brucei brucei* (AnTat 1.3A and pTSARib-0 transformants) or NHS-sensitive
25 *Trypanosoma brucei rhodesiense* ETat 1.2S, whereas an equivalent extract from control CHO cells did not (Fig. 4b,d). Significantly, the same lytic effect of apoL-I was obtained if foetal calf serum was used instead of apoL-I-depleted normal human serum (NHS) (Fig. 4b), and lysis was
30 not observed on normal human serum (NHS)-resistant cells (either *Trypanosoma brucei rhodesiense* ETat1.2R or *Trypanosoma brucei brucei* pTSARib-SRA) (Fig. 4c,d). Under the same conditions, recombinant apoL-I lacking the 343-398 C-terminal peptide still induced full lysis of normal human

serum (NHS)-sensitive trypanosomes, but also affected normal human serum(NHS)-resistant cells (Fig. 4b,c). Such an effect by an apoL-I mutant deficient in interaction with serum resistance associated protein (SRA) confirmed that
5 this interaction is involved in resistance to lysis.

These data show that apoL-I is the trypanolytic factor of normal human serum (NHS) and that serum resistance associated protein (SRA) neutralizes its activity, presumably through coiled-coil interaction.
10 Previous studies identified the lytic factor of NHS as haptoglobin-related protein (Hpr), another HDL-linked serum protein only found in primates ¹³, but this view was debated ^{2,14}. Moreover, the suggestion that Hpr might be necessary to allow uptake of the lytic particles into
15 trypanosomes ¹⁵, is contradicted by this observation that apoL-I kills trypanosomes in Hpr-free medium. The mechanism of lysis by apoL-I is not understood. Indeed, despite the evidence that lysis by normal human serum (NHS) is due to disruption of the lysosomal membrane ^{1,16}, these data show
20 that the fusogenic 343-355 peptide of apoL-I is not required. Resistance to lysis seemed due to SRA-mediated inhibition of the apoL-I lytic effect within the lysosome.

Interaction of serum resistance associated protein (SRA)
25 with in vitro synthesized apoL-I

[³⁵S]-apoL-I was produced by in vitro translation in reticulocyte lysates, using the apoL-I-pcDNA3.1/V5-His construct or mutant derivatives as template for transcription. All apoL polypeptides contained an in-
30 frame V5 tag at their C-terminus, and they were additionally flanked or not with a C-terminal His-tag, depending on the insertion of an in-frame stop codon between the two tag sequences. For interaction with His-SRA, apoL-I with the V5 tag alone was used. In this case, 5

μ l of [35 S]-apoL-I-containing reticulocyte lysate was incubated for 4 h at 4°C with 15 μ g of His-SRA in buffer A. Further treatment with Ni-NTA beads was performed as described above. The bound/unbound apoL-I was revealed by
5 autoradiography and quantitated by liquid scintillation counting after precipitation in 10% trichloroacetic acid.

Modelling

A 3D model of the apoL-I C-terminal/SRA N-terminal peptide complex was built using the Swissmodel and Swiss-PDB Viewer programs ¹⁹. The 3D structure of the coiled coil domain (aa 7-112) of the MiTat 1.2 VSG ⁷ (PDB code: 2VSG) was used as template. We made the assumption that the SRA N-terminal and the apoL-I C-terminal fragments
15 interact like the two VSG helices. The 31-79 sequence of SRA was aligned to residues 7 to 54 of the mature VSG (first helix), since that SRA fragment is the most similar to the latter domain of the VSG. The apoL-I 340-392 sequence was therefore aligned to residues 59-112 of the
20 variant surface glycoprotein (VSG) (second helix). The parallel (N-C versus N-C) and anti-parallel (N-C versus C-N) matchings were built. The resulting structures were minimised using Hyperchem 5.0 (Hypercube, Inc.), with the conjugate gradient method and AMBER force field. The lowest
25 energy structure was kept. The stereochemical quality of the model was checked with Procheck ²⁰: 98% of the residues were in the allowed regions of the Ramachandran plot. Molecular views were drawn with the WinMGM program ²¹.

30 Dissociation and reconstitution of trypanolytic activity of normal human serum (NHS)

Untreated, affinity-depleted and/or supplemented serum was added to HMI-9 medium containing 5% CHAPS, to a final concentration of 25%. Recombinant apoL-I

was used at physiological concentration (8 $\mu\text{g/ml}$). After 2h-incubation CHAPS was removed by extensive dialysis. The trypanosomes were seeded at $5 \cdot 10^5$ cells/ml. The results shown are representative of at least three separate
5 experiments, and they were confirmed *in vivo*: when directly injected into NMRI mice, the incubation mixtures tested as non-lytic or lytic led to detectable parasitaemia after two days or at least 10 days, respectively.

Trypanosomes from the *Trypanosoma brucei*
10 *rhodesiense* ETat 1.2R clone were purified from the buffy coat using PSGSA (3 mM NaH_2PO_4 , 50 mM Na_2HPO_4 , 44 mM NaCl, 100 mM sucrose, 83 mM glucose, 0.1 mM adenosine, pH8.0). The cells were washed three times in PSGSA and then resuspended at 10^9 /ml in TSC (150 mM NaCl, 1% CHAPS, 25 mM
15 Tris-HCl pH 7.5) containing leupeptin (30 $\mu\text{g/ml}$), PMSF (0.2 mM), E-64 (20 μM), TLCK (50 μM), aprotinin (10 $\mu\text{g/ml}$), TPCK (50 μM), pepstatin (10 $\mu\text{g/ml}$) and DNase I (1 $\mu\text{g/ml}$). The lysate was incubated on ice for 1 h with occasional shaking and then centrifuged at 20,000 g for 1 h at 4°C. The
20 supernatant was dialysed twice against 10 volumes of TSC prior to loading on to Concanavalin A-Sepharose (14 mg/ml resin). Prior to loading the supernatant was adjusted to 0.1 mM CaCl_2 and applied (0.2 ml/min) to a Con A column (3 ml bed volume) both equilibrated with TSC. The column was
25 washed with TSC before reversing the flow and eluting the bound glycoproteins using TSC containing either a mixture of chito-oligosaccharides that specifically compete with α -methylmannoside (0.3 M). Fractions (0.65 ml) were collected throughout and subjected to Western blot analysis using
30 anti-SRA antibodies.

The first experiments were designed to evaluate the role of apparent sequence abnormalities of SRA, namely the possible lack of N-terminal signal peptide

and the absence of two C-terminal cysteines, both features normally found in VSGs. Adding the sequence for the signal peptide of the variant surface glycoprotein (VSG) (aa 1 to 14) to serum resistance associated protein (SRA), or
5 conversely deleting this sequence from the variant surface glycoprotein (VSG), did not change their respective effect on the phenotype of transfected cells, namely resistance and sensitivity to normal human serum (NHS) for serum resistance associated protein (SRA) and variant surface
10 glycoprotein (VSG) respectively. Similarly, restoring the two C-terminal cysteines to serum resistance associated protein (SRA), as well as deleting these cysteines from the variant surface glycoprotein (VSG), did not change the effect of these proteins on resistance, namely resistance
15 and sensitivity, respectively. The regions encoding the N-terminal domain (aa 1-192 and 1-286 in serum resistance associated protein (SRA) and variant surface glycoprotein (VSG) respectively) were exchanged, generating variant surface glycoprotein (VSG)-SRA and serum resistance
20 associated protein (SRA)-VSG chimers. Only the SRA-VSG construct led to resistance. Another construct associating the variant surface glycoprotein (VSG) C-terminal domain to a longer fragment of serum resistance associated protein (SRA), up to aa 286, also led to resistance. Altogether
25 these data indicated that the C-terminal end of serum resistance associated protein (SRA), downstream from aa 192, is not required for resistance. This was confirmed by exchanging the second half of the C-terminal domains of the two proteins (aa 348-410 and 436-490 in serum resistance
30 associated protein (SRA) and variant surface glycoprotein (VSG) respectively), which conserved their respective ability to confer resistance. Inserting the surface-exposed region of the VSG (aa 287 to 367) downstream from residue 286 of SRA, thus, restoring a normal variant surface

glycoprotein (VSG) size to serum resistance associated protein (SRA), still allowed the latter to confer resistance to normal human serum (NHS). Conversely, deleting this region from the VSG did not allow it to
5 confer resistance to NHS. The possible involvement of the glycosylphosphatidylinositol (GPI) anchor was also evaluated. SRA was mutated in the three codons predicted to be essential for transamidation of the GPI to the protein (DSS). These amino acids were replaced by either three stop
10 codons or the HKR triplet that is not supposed to be recognized by the transamidation machinery. Thus, in the former case the serum resistance associated protein (SRA) precursor should lack the C-terminal hydrophobic tail normally exchanged with the GPI, whereas in the latter case
15 this hydrophobic tail should not be exchangeable with the GPI. Both gene constructs conferred full resistance to normal human serum (NHS), strongly suggesting that neither GPI nor GPI-anchoring is essential to confer resistance.

20 Fusogenic activity of the C-terminal region of apoL-I

Materials and Methods

Peptide synthesis. The following peptides were synthesized using Fmoc chemistry, purified by HPLC and checked by mass spectrometry: ApoL 345-355 : LALDVVYLVYES;
25 ApoL 340-362 : PVSFFLALDVVYLVYESKHLHEG; SRA 54-65 : ALAKINNLIKQ.

Liposome fusion assays. Large unilamellar vesicles (LUV) were prepared by the extrusion technique of Hope et al ²⁴ using an extruder (Lipex Biomembranes Inc,
30 Vancouver, Canada). In brief, dry lipid films that are mixtures in weight of 26.6 % phosphatidylcholine (PC), 26.6% sphingomyelin (SM), 26.6 % phosphatidylethanolamine (PE) and 20.2 % cholesterol were hydrated for 1h at 37°C. The resulting suspension was submitted to 5 successive

cycles of freezing and thawing, and thereafter extruded 10 times through 2 stacked polycarbonate filters (pore size 0.08 μm), under a nitrogen pressure of 20 bars. The concentration of the liposome suspensions was determined by phosphorus analysis ²⁵.

Lipid-mixing experiments. Mixing of liposome membranes was followed by measuring the fluorescence increase of R18 (octadecyl rhodamine chloride), a lipid soluble probe, occurring after the fusion of labeled and unlabeled liposomes. Labeled liposomes were obtained by incorporating R18 in the dry lipid film at a concentration 6.3 % of the total lipid weight. Labeled and unlabeled liposomes were mixed at a weight ratio 1:4 at a final concentration of 50 μM in buffer. Depending on pH conditions, different buffers were used: pH 7.4: 10mM Tris, 150 mM NaCl, 0.01% EDTA, 1 mM NaN_3 ; pH 6: 10mM acetate, 150 mM NaCl, 0.01% EDTA, 1 mM NaN_3 ; pH 4: 100 mM acetic acid, 57 mM acetate. Peptides were dissolved in trifluoroethanol (TFE). The final TFE percentage was 1.6 % vol. In a control experiment, the same volume of TFE alone was added to the liposome mixture. Fluorescence was recorded at room temperature (λ_{exc} : 560 nm, λ_{em} : 590 nm) on an LS-50B Perkin Elmer fluorimeter.

The 345-355 peptide of apoL-I has hydrophobic properties close to those of tilted peptides ^{12,22}. The latter were shown to have lipid-destabilising properties and so we tested the apoL-I peptide on liposomes. This peptide clearly increased the fluorescence of the lipophilic R18 probe due to its dilution after lipid fusion. The process was dose-dependent and was the most efficient at 0.1 peptide to lipid molar ratio. Under the same conditions, the 340-362 longer peptide was even more fusogenic. The highest fluorescence increase was seen at pH

4, but was also significant at pH 6 or 7.4 (data not shown).

When the latter conditions were tested, the fusion occurred on a longer time scale (one hour instead of 5 15 minutes). Finally, the lipid composition of the liposomes did not significantly affect the fusogenic properties of the peptide (data not shown).

Interaction with the 54-65 peptide of serum resistance associated protein (SRA) clearly affected the 10 fusion induced by the 345-355 peptide from apoL-I. Increasing the time of preincubation of the peptides increased fusion inhibition. This was also true for the 340-362 peptide of apoL-I (data not shown). The decrease in R18 fluorescence following the co-incubation of serum 15 resistance associated protein (SRA) and apoL-I peptides was indicative of a direct interaction between these regions, as already observed for other peptides ²³.

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CLAIMS

1. A pharmaceutical composition comprising an adequate pharmaceutical carrier or diluent and a sufficient amount of an element selected from the group consisting of the apolipoprotein L-I, a pharmaceutical active fragment thereof, a polynucleotide encoding said polypeptide, a cell transformed by said polynucleotide or an inhibitor directed against said apolipoprotein L-I.
2. The pharmaceutical composition according to claim 1, wherein the apolipoprotein L-I is the human apolipoprotein L-I corresponding to the sequence SEQ.ID.N°1 or an homologue polypeptide.
3. The pharmaceutical composition of claim 2, wherein the fragment of the human apolipoprotein L-I is selected from the group consisting of the sequence starting from the amino acid 1 up to the amino acid 342, the sequence starting from the amino acid 343 to the amino acid 398, the sequence starting from the amino acid 340 up to the amino acid 362 and the sequence starting from the amino acid 356 up to the amino acid 398 of the human polypeptide apolipoprotein sequence SEQ.ID.N° 1.
4. The pharmaceutical composition according to claim 1, wherein the inhibitor directed against apolipoprotein L-I is the trypanosoma polypeptide SRA or a pharmaceutical active fragment thereof or any molecule which mimic the interaction between the polypeptide SRA and the apolipoprotein L-I.
5. The pharmaceutical composition according to claim 4, wherein the pharmaceutical active fragment of the Trypanosoma polypeptide SRA is the fragment of said polypeptide, which interacts specifically with apolipoprotein L-I.

6. The pharmaceutical composition according to claim 4, wherein the molecule which mimic the interaction between the polypeptide SRA and the apolipoprotein L-I is an antibody (or a hyper variable
5 portion thereof) directed against apolipoprotein L-I, preferably, an antibody (or a hyper variable portion thereof) directed against the terminal fragment of apolipoprotein L-I, which interacts with the Trypanosoma polypeptide SRA.

10 7. The pharmaceutical composition according to claim 1, wherein the inhibitor is an anti-idiotypic antibody or a hyper variable portion thereof directed against the anti-apolipoprotein L-I antibody (or a hyper variable portion thereof).

15 8. Use of the pharmaceutical composition according to any of the preceding claims 1 to 7, for the manufacture of a medicament for the treatment and/or the prevention of diseases induced in mammals (including the human) by Trypanosoma, especially African Trypanosoma.

20 9. Use according to claim 8, wherein the Trypanosoma are selected from the group consisting of *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense* and *Trypanosoma gambiense*.

25 10. Use according to claim 8 or 9 for the treatment of Nagana induced in bovidae by *Trypanosoma brucei brucei*.

30 11. A diagnostic kit comprising an element selected from the group consisting of the apolipoprotein L-I, a fragment thereof, a polynucleotide encoding said apolipoprotein L-I or an inhibitor directed against said apolipoprotein.

12. The diagnostic kit of claim 11, wherein the apolipoprotein L-I is the human apolipoprotein L-I or an homologue polypeptide.

13. The diagnostic kit according to claim 11 or 12, wherein said inhibitor is the trypanosoma polypeptide SRA or a fragment thereof which interacts with said apolipoprotein L-I or an antibody (or a hyper variable
5 portion thereof) directed against said apolipoprotein L-I.

14. A non-human genetically modified mammal which is expressing a polynucleotide encoding the apolipoprotein L-I (preferably the human polypeptide apolipoprotein L-I or an homologue of said polypeptide) or
10 an active pharmaceutical fragment thereof and wherein the mammal is resistant or tolerant to diseases induced by Trypanosoma, especially *Trypanosoma brucei brucei* (Nagana).

15. The mammal according to claim 14 which is a genetically modified bovidae.

15 16. The mammal according to claim 14 or 15, wherein the pharmaceutical active fragment of apolipoprotein is the sequence starting from the amino acid 1 up to the amino acid 342.

17. A solid support such as a chromatographic
20 column comprising, bound to the surface of said solid support, an inhibitor (preferably an antibody, the SRA polypeptide or a fragment thereof) directed against the apolipoprotein L-I and used for the recovery of said apolipoprotein L-I from a mammal, preferably a human body
25 sample.

18. A method for the recovering of apolipoprotein L-I polypeptide from a mammal, preferably a human body sample (such as human serum), said method comprising the steps of:

- 30 - putting into contact said sample with the solid support of claim 17,
- eluting the contaminant of said sample and the apolipoprotein L-I bound to the inhibitor.

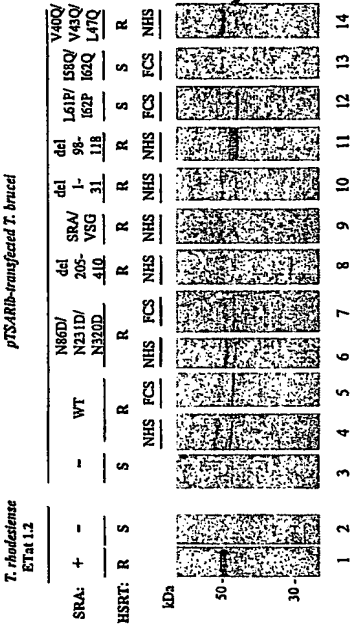


Figure 1

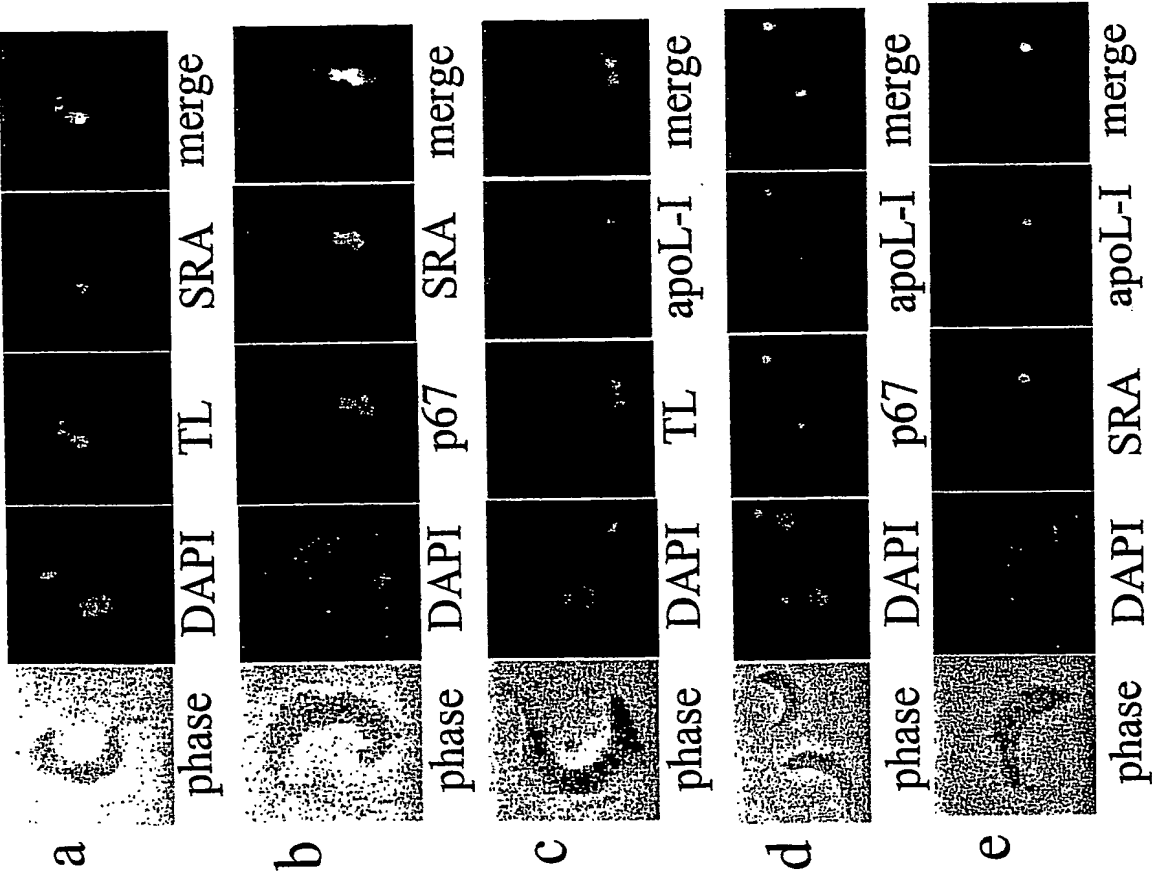


Figure 2

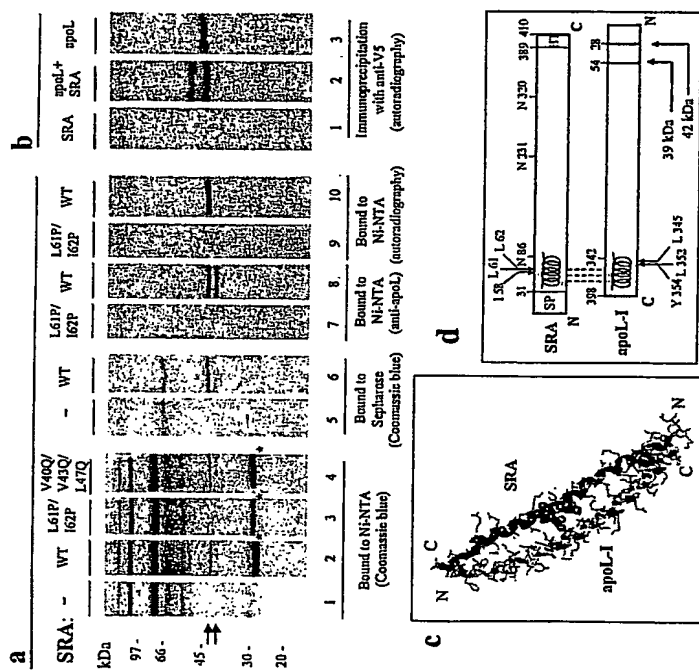
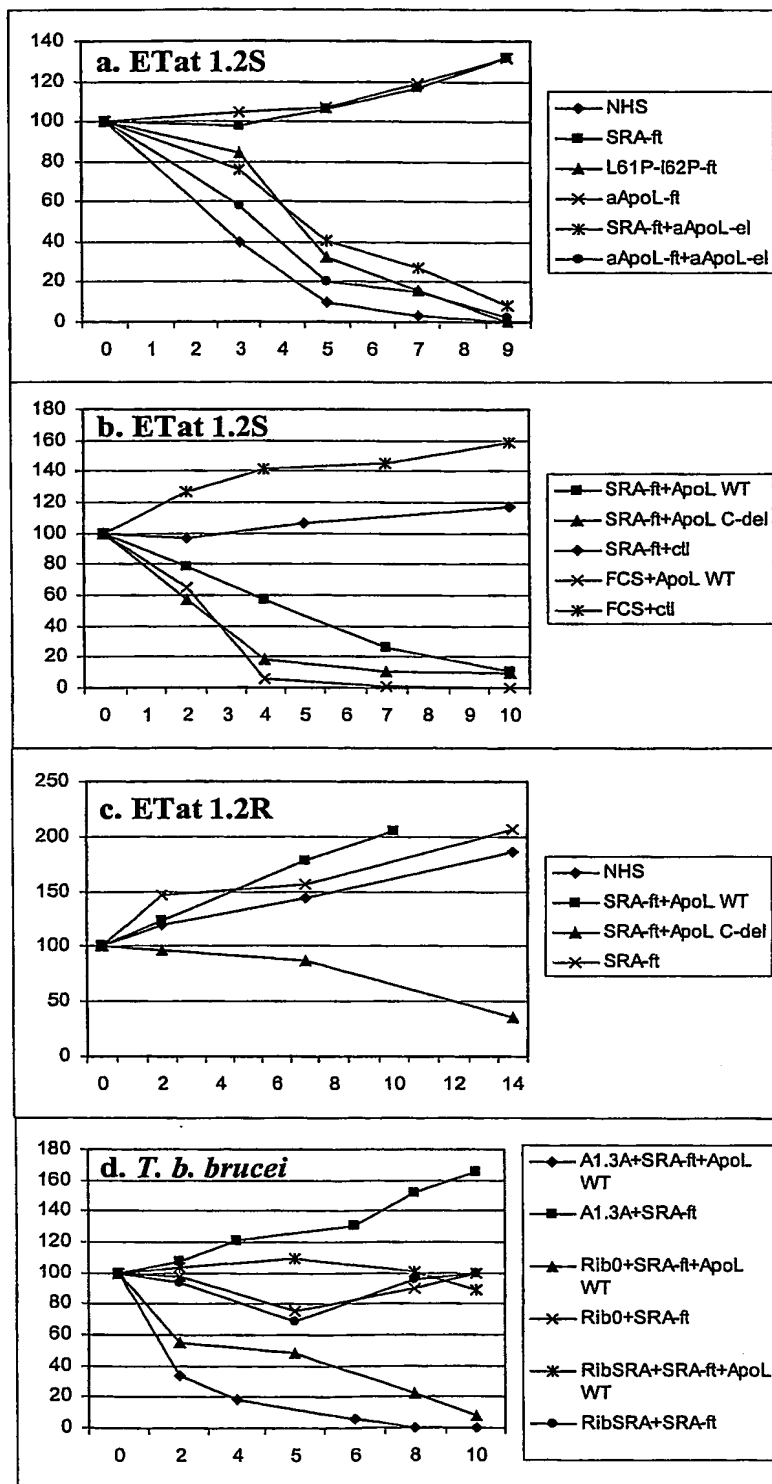


Figure 3

Trypanosome number (%)



Time (hours)

Figure 4

MEGAALLRVSVLCIWMSALFLGVRVRAEEAGARVQQNVPSGTDGDPQSKPLGDWAA
GTMDPESSIFIEDAIKYFKEKVSIGNLLLLLTDNEAWNGFVAAAELPRNEADELRKALDN
LARQMIMKDKNWHDKGQQYRNWFLKEFPRLKSKLEDNIRRLRALADGVQKVHKGTTI
ANVVSGSLSISSGILTLVGMGLAPFTEGGSLVLEPGMELGITAALTGITSSTIDYGKKWW
TQAQAHDLVIKSLDKLKEVKEFLGENISNFLSLAGNTYQLTRGIGKDIRALRRARANLQS
VPHASASRPRVTEPISAESGEQVERVNEPSILEMSRGVKLTDVAPVSFFLVLDVVYLVYES
KHLHEGAKSETAEELKKVAQELEEKLNLNNNYKILQADQEL

Figure 5a

Sequence ApoL-I gene in pCDNA3-V5HIS (from RT-PCR on HepG2 RNA)

AAGCTTGGTACC

ATGGAGGGAGCTGCTTTGCTGAGAGTCTCTGTCCTCTGCATCTGGATGAGTGCACTTT
TCCTTGGTGTGGGAGTGAGGGCAGAGGAAGCTGGAGCGAGGGTGCAACAAAACGTT
CCAAGTGGGACAGATACTGGAGATCCTCAAAGTAAGCCCCTCGGTGACTGGGCTGCT
GGCACCATGGACCCAGAGAGCAGTATCTTTATTGAGGATGCCATTAAGTATTTCAAG
GAAAAAGTGAGCATAACAGAATCTGCTACTCCTGCTGACTGATAATGAGGCCTGGAAC
GGATTCGTGGCTGCTGCTGAACTGCCCAGGAATGAGGCAGATGAGCTCCGTAAAGCT
CTGGACAACCTTGCAAGACAAATGATCATGAAAGACAAAACTGGCACGATAAAGG
CCAGCAGTACAGAACTGGTTTCTGAAAGAGTTTCCTCGGTTGAAAAGTAAGCTTGA
GGATAACATAAGAAGGCTCCGTGCCCTTGCAAGATGGGGTTCAGAAGGTCCACAAAG
GCACCACCATCGCCAATGTGGTGTCTGGCTCTCTCAGCATTTCTCTGGCATCCTGAC
CCTCGTCGGCATGGGTCTGGCACCTTCACAGAGGGAGGCAGCCTTGTACTCTTGGA
ACCTGGGATGGAGTTGGGAATCACAGCAGCTTTGACCGGGATTACCAGCAGTACCAT
AGACTACGGAAAGAAGTGGTGGACACAAGCCCAAGCCACGACCTGGTCATCAAAA
GCCTTGACAAATTGAAGGAGGTGAAGGAGTTTTTGGGTGAGAACATATCCAACCTTC
TTTCCTTAGCTGGCAATACTTACCAACTCACACGAGGCATTGGGAAGGACATCCGTG
CCCTCAGACGAGCCAGAGCCAATCTTCAGTCAGTACCGCATGCCTCAGCCTCACGCC
CCCGGGTCACTGAGCCAATCTCAGCTGAAAGCGGTGAACAGGTGGAGAGAGTTAAT
GAACCCAGCATCCTGGAAATGAGCAGAGGAGTCAAGCTCACGGATGTGGCCCCCTGT
AAGCTTCTTTCTTGTGCTGGATGTAGTCTACCTCGTGTACGAATCAAAGCACTTACAT
GAGGGGGCAAAGTCAGAGACAGCTGAGGAGCTGAAGAAGGTGGCTCAGGAGCTGG
AGGAGAAGCTAAACATTCTCAACAATAATTATAAGATTCTGCAGGCGGACCAAGAA
CTG
CTCGAGTCTAGAGGGCCCTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCG
ATTCTACGCGTACCGGTCATCATCACCATCACCATTGA

GTTTAAACCCGCTGATCAGC

Figure 5b

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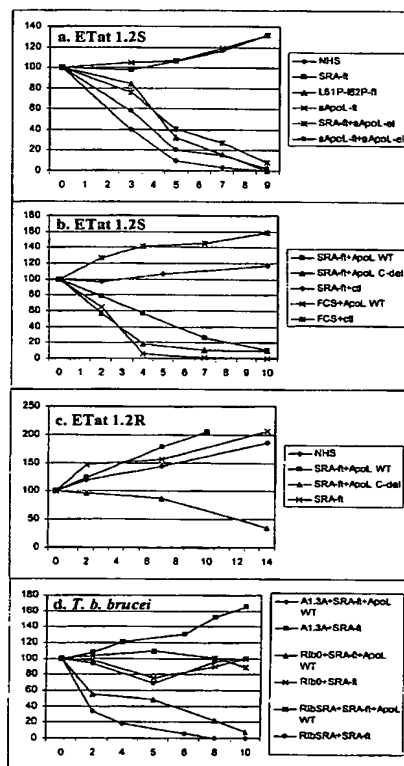
(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: **APOLIPOPROTEIN L-I FOR THE TREATMENT OR DIAGNOSIS OF PRYPANOSOMALDISEASES**

Trypanosome number (%)



Time (hours)

(57) Abstract: The present invention is related to a composition comprising apolipoprotein L-I, the use of apolipoprotein L-I or a derivated polypeptide for the diagnostic, the treatment and/or the prevention of diseases induced in mammals by Trypanosoma. Another aspect is related to a transgenic non-human mammal comprising a polynucleotide expressing said apolipoprotein or a derivated polypeptide and which is tolerant or resistant to said Trypanosoma infection.

WO 2004/012757 A3



European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(88) Date of publication of the international search report:
15 April 2004

Published:

- with international search report
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No.
P 03/00131

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/17 A61P33/02 A01K67/027 G01N33/92

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>TYTLER E M ET AL: "Reconstitution of the trypanolytic factor from components of a subspecies of human high-density lipoproteins."</p> <p>MOLECULAR AND BIOCHEMICAL PARASITOLOGY. NETHERLANDS JAN 1995, vol. 69, no. 1, January 1995 (1995-01), pages 9-17, XP001155959</p> <p>ISSN: 0166-6851</p> <p>abstract</p> <p>Introduction and Discussion particularly the final paragraph</p> <p style="text-align: center;">----- -/-</p>	1-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

17 February 2004

Date of mailing of the international search report

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Pilling, S

INTERNATIONAL SEARCH REPORT

International Application No

BE 03/00131

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MILNER J D ET AL: "Expression and localization of serum resistance associated protein in Trypanosoma brucei rhodesiense." MOLECULAR AND BIOCHEMICAL PARASITOLOGY. NETHERLANDS 30 NOV 1999, vol. 104, no. 2, 30 November 1999 (1999-11-30), pages 271-283, XP001155961 ISSN: 0166-6851 abstract introduction particularly the first paragraph and the Discussion particularly the last paragraph</p>	1-10
A	<p>DUCHATEAU P N ET AL: "Apolipoprotein L gene family: Tissue-specific expression, splicing, promoter regions; discovery of a new gene" JOURNAL OF LIPID RESEARCH, BETHESDA, MD, US, vol. 42, no. 4, April 2001 (2001-04), pages 620-630, XP002247906 ISSN: 0022-2275 the whole document</p>	1-10
A	<p>PAGE N M ET AL: "The human apolipoprotein L gene cluster: identification, classification, and sites of distribution." GENOMICS. UNITED STATES 15 MAY 2001, vol. 74, no. 1, 15 May 2001 (2001-05-15), pages 71-78, XP001155958 ISSN: 0888-7543 the whole document</p>	1-10
P,X	<p>VANHAMME LUC ET AL: "Apolipoprotein L-I is the trypanosome lytic factor of human serum." NATURE. ENGLAND 6 MAR 2003, vol. 422, no. 6927, 6 March 2003 (2003-03-06), pages 83-87, XP001155956 ISSN: 0028-0836 abstract</p>	1-10
A	<p>US 5 767 337 A (ROSES ALLEN D ET AL) 16 June 1998 (1998-06-16) the whole document</p>	14-16
A	<p>WO 99/35241 A (EMMANUEL FLORENCE ; HOUEBINE LOUIS MARIE (FR); ROUY DIDIER (FR); DENE) 15 July 1999 (1999-07-15) the whole document</p>	14-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BE 03/00131

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
1-10, 14-16
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-10

pharmaceutical compositions/uses comprising apolipoprotein L-I (apoL-I), an active fragment thereof, a polynucleotide encoding apoL-I, a cell transformed with the latter polynucleotide or an apoL-I inhibitor

2. claims: 11-13

diagnostic kits comprising apoL-I, a fragment thereof, a polynucleotide encoding apoL-I or an apoL-I inhibitor

3. claims: 14-16

non-human genetically modified mammals which express a polynucleotide encoding apoL-I or a fragment thereof and are resistant to Trypanosoma diseases

4. claims: 17,18

solid supports/methods for recovering apoL-I from a mammal using an immobilised apoL-I inhibitor

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/JP98/03/00131

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5767337	A	16-06-1998	AU 720830 B2	15-06-2000
			AU 6485096 A	26-02-1997
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			EP 0842271 A1	20-05-1998
			JP 11510052 T	07-09-1999
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			CA 2316808 A1	15-07-1999
			EP 1049767 A1	08-11-2000
			JP 2002500039 T	08-01-2002
			WO 9935241 A1	15-07-1999
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